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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of DONALD S. ANSON ET AL. Attorney Docket: 604-8

Serial Number: 06/839,215 Group Art Unit: 183

Filed: March 13, 1986 Examiner: J. Kushan

For: FACTOR IX PROTEIN

DECLARATION UNDER RULE 132

PROFESSOR GEORGE GOW BROWNLEE F.R.S. declares as follows:

1. I am the E.P. Abraham Professor of Chemical Pathology at the Sir William Dunn School of Pathology, University of Oxford, England and one of the inventors of the above-identified patent application. I have read the letters dated 19th May and 24th June 1988 from the United States Patent and Trademark Office.

2. In the body, factor IX is synthesised in hepatocytes (liver cells) where there is first produced a precursor protein of factor IX. The precursor is not active in the blood-clotting pathway. It undergoes at least two stages of protein processing involving peptide cleavage, as well as three distinct types of post-translational modification, before secretion into the bloodstream as a 415 amino acid long mature, biologically active glycoprotein.

The post-translational modifications are the carboxylation of twelve glutamic acid residues by an enzyme (carboxylase) which requires vitamin K (AAI), the addition of several carbohydrate residues (AAJ) and the beta-hydroxylation of a single aspartic acid residue (AAI, AAL). The first two modifications were known to be required for activity, the third

(AAK, page 5703)
was uncertain. Additionally, "prepeptide" and "propeptide" sequences are removed as part of the processing of the precursor to give the mature, active protein. Because of the complex and specialised nature of the processing and these modifications, it seemed to me probable at the time of the invention that the expression of fully active human factor IX (i.e. having the same activity as that obtained from blood plasma) derived from human factor IX DNA clones would present great problems.

3. The patent specification shows two routes, both in mammalian cells, for producing biologically active factor IX, which is at least 90% as active as factor IX derived from blood, in its biological properties of clotting blood.

At the time of making this invention, I had high hopes that it would be possible to produce recombinant factor IX protein from the cloned gene, but I feared that the recombinant material would be inactive in its property of clotting blood. My poor expectations were based on my knowledge of the post-translational modifications required, particularly (a) the conversion of 12 glutamic acids to γ carboxyglutamyl residues by vitamin K-dependent carboxylase and (b) the beta-hydroxylation. As regards the γ-carboxylation, I did not know whether the vitamin K-dependent carboxylase would work satisfactorily in tissue cell culture, especially whether high enough concentrations would be present. The β-hydroxylation of the aspartic acid residue was still more of a problem since it was not certain that it was required and if so which enzyme brought it about. It seemed to me unlikely at that time that both these enzyme systems would be functioning fully and normally <u>in vitro</u> in the hepatoma cell

line H4-II-E-C3 (Example 1). Although the hepatoma cell line H4-II-E-C3 was known to secrete prothrombin, it did not follow that it would necessarily secrete factor IX. It was not known whether there would be sufficient γ-carboxylation to produce biologically active factor IX, nor was it known whether the cells would be able to carry out beta-hydroxylation of factor IX precursor as this is a post- translational modification which factor IX and prothrombin do not have in common (AAI, AAL). Nor does it follow from the known properties of other hepatomas. Indeed, it was well known that hepatomas are highly dedifferentiated and lose hepatocyte functions. For example, B.B. Knowles, C.C. Howe and D.P. Aden, Science 209 497-499 (1980) describe the protein-synthesising abilities of three human hepatomas HepG2, HepG3, and PLC/PRF/5. None of these lines produced pre-albumin or hemopexin which are made by normal hepatocytes and they also lacked ability to produce certain other proteins normally found in hepatocytes. See Table 1 including the footnotes, and page 498. In fact, my co-inventor Dr. D.S. Anson and I tried using human hepatomas HepG2 and HepG3 for the purposes of this invention, but they failed to secrete human factor IX, presumably also because of dedifferentiation.

It was also unlikely that the canine cell line "MDCK" used in Examples 2 and 3 would be a suitable cell line for the production of fully biologically active factor IX as this cell line derives from kidney cells - a tissue not believed to produce factor IX in vivo. In fact, the only reason that I and my co-inventors tried the unlikely experiment of producing factor IX in such cells was because of its use in my laboratory by one of the inventors for another

project totally unrelated to factor IX. Specifically, my co-inventor Dr. I.J. Jones was using MDCK cells to carry out experiments with influenza virus, as this is one of the few cell lines that can be used in vitro to produce progeny influenza virus. Most cell lines, for example fibroblasts or hepatomas, will support an initial influenza virus infection but this aborts and no progeny virus is produced. The propagation of influenza virus and production of factor IX by cells are, of course, totally unrelated.

4. I believe, therefore, that it was not predictable or expected that a fully (or near-fully) biologically active factor IX protein could be obtained by a recombinant DNA method.

5. I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such wilful false statements may jeopardize the validity of the application or any patent issued thereon.

G.G. Brownlee

GEORGE GOW BROWNLEE

10th November 1988

Date